HERBICOLINS—NEW PEPTIDE ANTIBIOTICS FROM *ERWINIA HERBICOLA*

G. WINKELMANN

Institut für Biologie I, Mikrobiologie I, Universität Tübingen,
D-7400 Tübingen, Auf der Morgenstelle 1, GFR

R. LUPP and G. JUNG

Institut für Organische Chemie, Universität Tübingen,
D-7400 Tübingen, Auf der Morgenstelle 18, GFR

(Received for publication January 25, 1980)

Erwinia herbicola (strain A 111) produces two acylated peptide antibiotics herbicolins A and B. Isolation of herbicolins was performed by adsorption on a polystyrol adsorbent followed by elution with methanol. Further purification was achieved by gel filtration on Sephadex LH-20, counter-current distribution or by TLC. Herbicolin A was chemically characterized, containing 2 glycines, 1 L-threonine, 1 D-*allo*-threonine, 1 D-glutamic acid, 1 D-leucine, 1 L-arginine and β-hydroxy myristic acid. Herbicolins A and B are inactive against bacteria, but highly active against yeasts and filamentous fungi.

During our screening program for new antifungal agents the bacterial strain A 111, later identified as *Erwinia herbicola*, was found to produce two peptide antibiotics, herbicolins A and B, when grown in carbohydrate-rich media[1]. This paper describes the fermentation, isolation and some physical, chemical and biological properties of herbicolin A.

Fermentation

Fermentation of *Erwinia herbicola* (A 111) was performed in a chemically defined medium containing per liter 7 g K_2HPO_4, 3 g KH_2PO_4, 0.5 g tri-Na-citrate·2 H_2O, 0.1 g $MgSO_4$·7 H_2O, 2 g $(NH_4)_2SO_4$, (pH 6.8) and 20 g glucose (autoclaved separately). Ten liters fermentation broth containing herbicolins were obtained by inoculating five shake flasks (containing 2 liters of medium) each with 100 ml of a 24-hour preculture (1 liter) containing the same medium. The culture flasks were incubated on a rotary shaker (120 rpm, 22°C) for 3 days.

Assay Procedure

The fermentation broth was tested for herbicolin production by TLC (Silica gel-60 plates, E. Merck, Darmstadt, Germany) using 1-butanol - acetic acid - water (4 : 1 : 1) as a solvent system for development. Herbicolin spots were detected by treating with gaseous chlorine and subsequent spraying with 4,4′-tetramethyldiamino-diphenylmethane[2].

In addition a plate diffusion test on agar seeded with conidiospores of *Neurospora crassa*, was performed to control the biological activity.

Isolation

Fermentation broth containing herbicolins was centrifuged at 6,000 *g* for 20 minutes in a Sorvall

centrifuge (Rotor GSA) or by continuous separation (Rotor SZ-14 GK). The centrifugate was then passed through a Servachrom XAD-2 column (Serva, Heidelberg, Germany). After washing with two volumes of distilled water and one volume acetone - water (1: 1) the herbicolins were desorbed with one volume methanol.

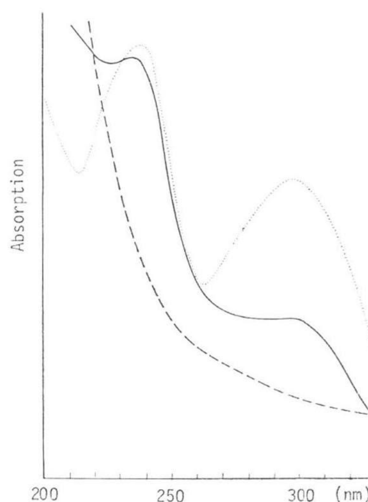
The eluate was concentrated under vacuum (50 ~ 60°C) and further purified by counter-current distribution using the solvent system 1-butanol - ethylacetate - water (1: 1: 1). On a CRAIG apparatus (Boy 505, Labortec) with 140 transfers (2 ml elements) pure herbicolin A was obtained in the tubes 60 ~ 90. Purification of herbicolin A was also achieved by gel filtration on Sephadex LH-20 using methanol as eluting solvent. The herbicolin A containing solution was evaporated or lyophilized yielding a white fluffy material. Herbicolin A crystallyzed from saturated methanolic solutions as small needles with a rectangular cross section.

Physico-chemical Characterization

Herbicolin A is soluble in lower alcohols, such as methanol, ethanol, propanol and butanol and also in mixtures of these alcohols with water and to a minor extent in pure water. It is less soluble in ethylacetate, chloroform, dichloromethane, and insoluble in ether and light petroleum. Herbicolins A and B can be separated by TLC on silica gel using 1-butanol - acetic acid - water or chloroform - methanol - water mixtures as solvent systems (Table 1). Herbicolin spots were ninhydrin negative, but revealed a positive reaction with chlorine/4, 4′-tetramethyldiamino-diphenylmethane[2], indicating the absence of free amino groups and the presence of peptide bonds. Treating with diazomethane did not change the chromatographic behavior indicating the absence of free carboxyl groups

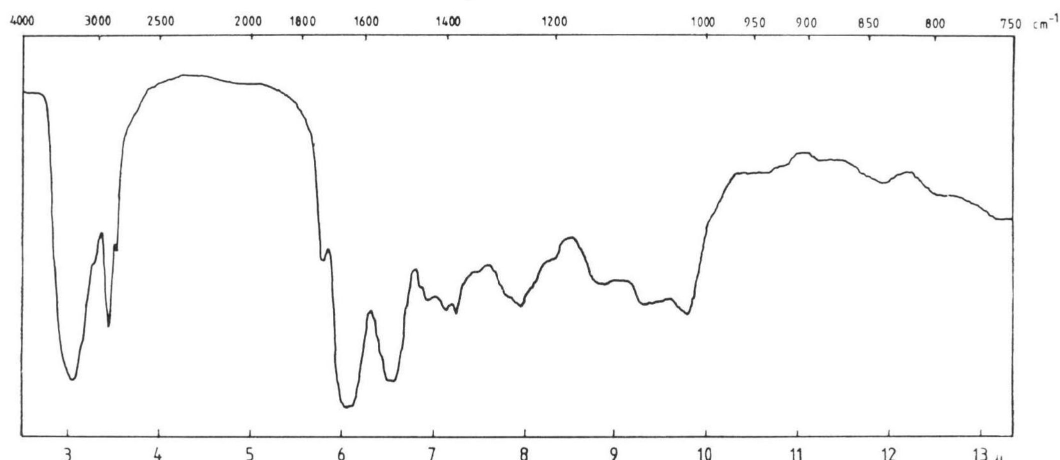
The UV spectrum of purified herbicolin A revealed no absorption maxima between 220 ~ 350 nm, but condensation with 1, 3-diketones led to a N^{δ}-(4,6-dimethylpyrimidyl-2)-L-ornithine derivative (λ_{max}=240 nm, and 300 nm)[3], indicating the presence of arginine residues (Fig. 1). The IR spectrum (Fig. 2) showed pronounced amide I and amide II stretch bands (1500 ~ 1700 cm^{-1}). Total hydrolysis of purified herbicolin A with 6 N HCl in sealed tubes at 110°C for 24 hours yielded five amino acids, when analysed in an amino acid analyzer: glycine, threonine, leucine, glutamic acid and arginine in a ratio 2: 2: 1: 1: 1. Gas chromatographic determination of the absolute configuration of the amino acids on the chiral phase *N*-propionyl-L-valine-*tert.* butylamide polysiloxan[4] revealed the presence of glycine, L-threonine, D-*allo*-threonine, D-glutamic acid, D-leucine and L-arginine (Fig. 3), indicating the presence of a heptapeptide portion. The circular dichroism spectrum (Fig. 4), however, excluded the presence of an α-helical conformation.

Fig. 1. Ultraviolet absorption spectrum of herbicolin A (------), N^{δ}-(4,6-dimethylpyrimidyl-2)ornithine (....) and N^{δ}-(4,6-dimethylpyrimidyl-2)-ornithyl-herbicolin A (——).
Solvent: methanol.



Boiling with anhydrous methanolic HCl for 60 minutes and extracting with hexane yielded

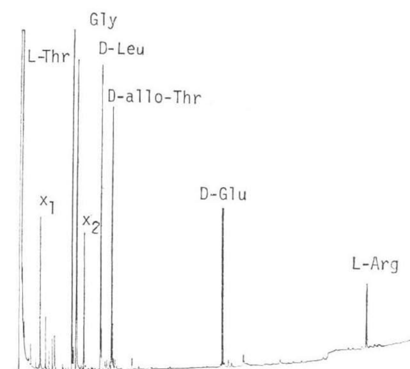
Fig. 2. Infrared spectrum of herbicolin A (KBr).



fatty acid methylesters. Gas chromatographic separation on OV-1 revealed, besides several minor peaks, one main peak with a retention volume equivalent to β-hydroxymyristic acid methylester (Fig. 5). The mass spectrum of this main peak obtained by GLC/MS confirmed the structural identity with a standard sample (Applied Science Europe B. V., Holland) indicating the presence of β-hydroxymyristic acid in herbicolin A (Fig. 6). The ^{13}C NMR spectrum of herbicolin A in CD_3OD is shown in Fig. 7. The ^{13}C NMR signals of the seven amino acids and of β-hydroxymyristic acid could be assigned by comparision with known data of these residues. Exact assignments of all signals will be published in a succeeding paper, when all constituents are determined.

Fig. 3. Gas chromatogram of an esterified and acylated amino acid hydrolysate of herbicolin A on a chiral phase (*N*-propionyl-L-valine-tert. butylamide-polysiloxan[4]).

Detector: FID, carrier gas: hydrogen, temperature program: 90~200°C, 4°C/min. Peaks x_1 and x_2 seem to arise from hydroxy alkanoic acids.



Biological Properties

As illustrated in the antimicrobial spectrum (Table 2), herbicolin A exhibited no activity against the following bacteria: *Bacillus subtilis* ATCC 6633, *Enterobacter cloacae* ATCC 13047, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27736, *Mycoplasma laidlawii*, *Proteus mirabilis*, *Proteus vulgaris* ATCC 6380, *Pseudomonas aeruginosa* ATCC 9721, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus pyogenes* ATCC 10389. The bacteria were grown on MUELLER-HINTON agar (Oxoid). In contrast all yeasts and filamentous fungi tested were sensitive to herbicolin A at concentration of 10 μg/disc in the plate diffusion test on yeast extract-malt extract-agar (4 g yeast extract, 4 g glucose and 10 g malt extract per liter). In a serial dilution test with *Neurospora*

Fig. 4. Circular dichroism spectrum of herbicolin A in methanol solution.
The dichroic absorption is refered to a concentration of 1 g/100 ml and an optical path length of 1 cm.

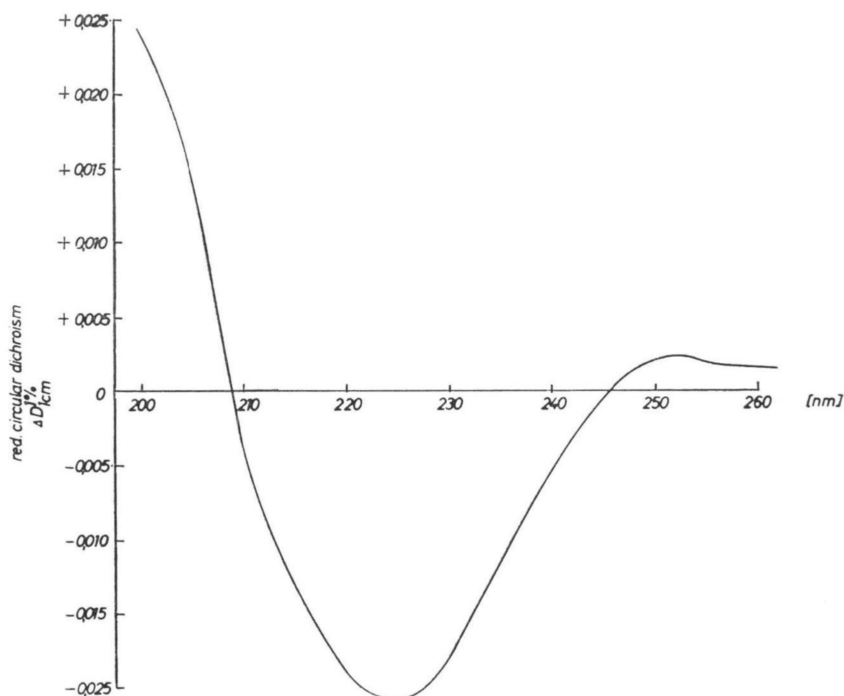
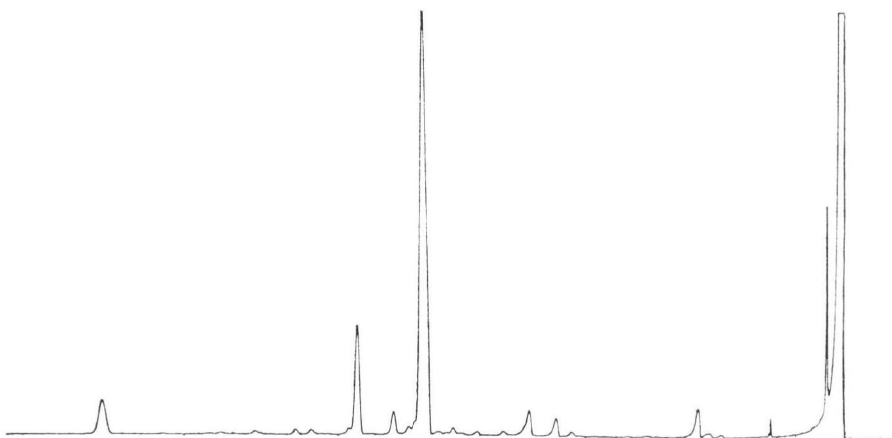


Fig. 5. Gas chromatogram of fatty acid methyl esters obtained from herbicolin A on OV-1, temperature 100~200°C, 6°C/min.



crassa the minimal inhibition concentration was 0.25 μg/ml in a chemically defined medium containing salts, asparagine and glucose. Inhibition was reversed by sterols and cardiolipin. Other eucaryotic cells, such as microalgae, protozoa and erythrocytes were also affected by herbicolin A. These activities will be reported elsewhere.

Fig. 6. Mass spectrum of β-hydroxymyristic acid methyl ester.
Reference sample (above) and the isolated sample (below).

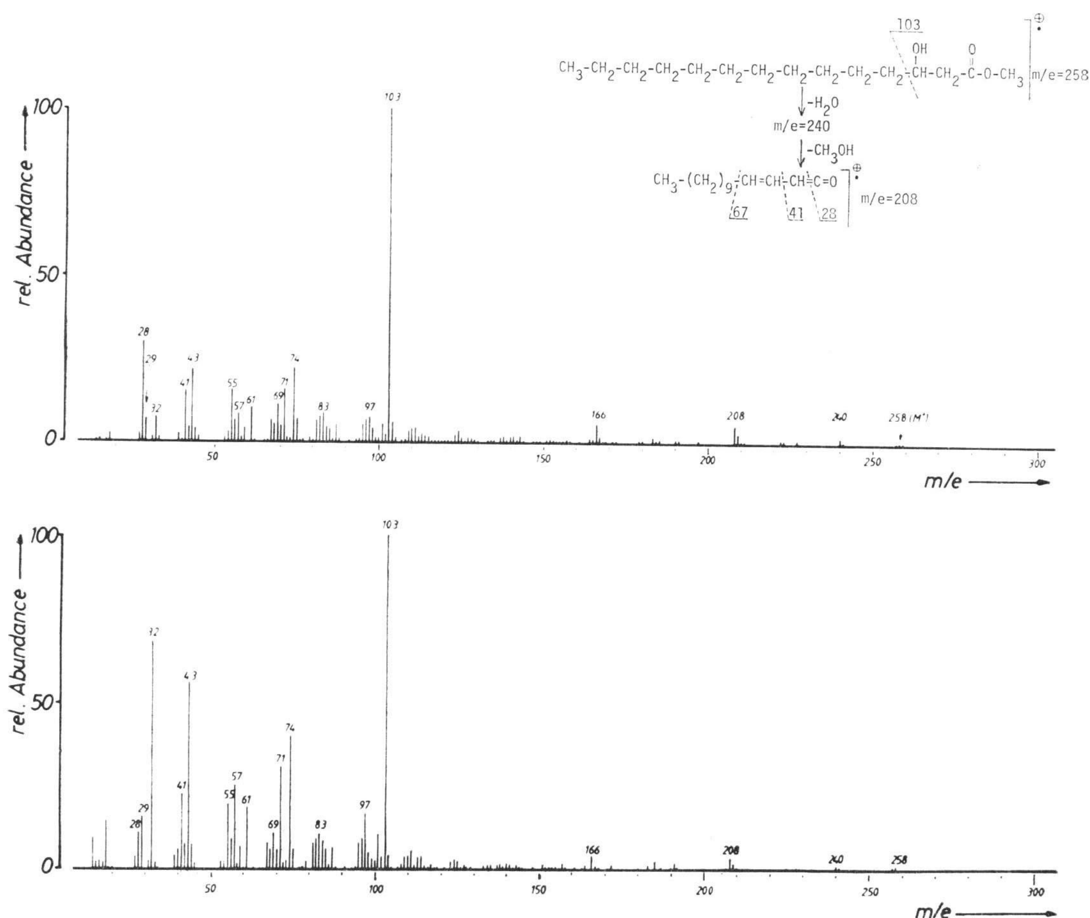


Table 1. Rf-values of herbicolins A and B on thin-layer plates (Merck, silica gel plates).
The spots were detected by chlorine/4,4′-tetramethyldiamino-diphenylmethane.

Solvent system	Rf	
	Herbicolin A	Herbicolin B
1-Butanol - acetic acid - water (4: 1: 1)	0.20	0.35
1-Butanol - acetic acid - water (3: 1: 1)	0.34	0.44
Chloroform - methanol - water (65: 25: 4)	0.05	0.22
Chloroform - methanol - acetic acid - water (25: 15: 4: 2)	0.21	0.72

Acknowledgements

We wish to thank W. JUNG and M. BUSSE (Südd. Versuchs und Forschungsanstalt für Milchwirtschaft, Weihenstephan) and U. ULLMANN (Hygiene Institut, Tübingen) for their assistance in determining and testing some bacterial strains and GRAME NICHOLSON and GERLINDE REICHLE for valuable technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 76).

Fig. 7. ^{13}C NMR spectrum (20.1 MHz) of herbicolin A in $^{12}CD_3OD$.

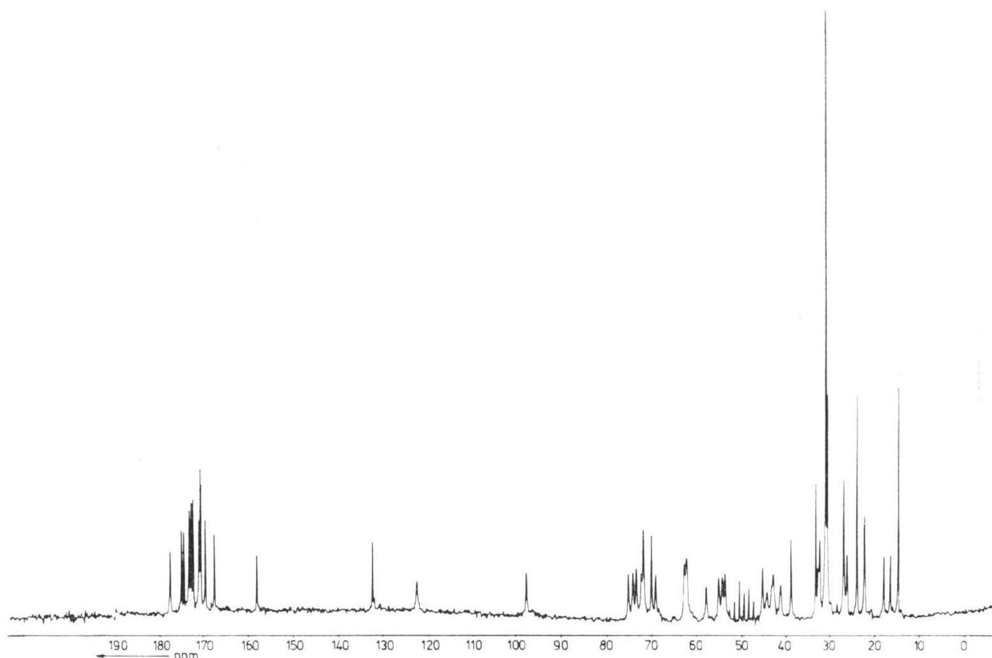


Table 2. Antimicrobial activity of herbicolin A against fungi and bacteria, determined by the plate diffusion test using 10 μg herbicolin A per filter disc.

Aspergillus fumigatus	+	*Schizonella melanogramma*	+	*Klebsiella pneumoniae* ATCC 27736	—
Aspergillus melleus	+	*Urocystis occulta*	+	*Myoplasma laidlawii*	—
Botrytis cinerea	+	*Ustilago nuda*	+	*Proteus mirabilis*	—
Fusarium dimerum	+	*Trichophyton rubrum*	+	*Proteus vulgaris* ATCC 6380	—
Neurospora crassa	+	*Epidermophyton floccosum*	+	*Pseudomonas aeruginosa* ATCC 9721	—
Cryptococcus melibiosum	+	*Bacillus subtilis* ATCC 6633	—	*Staphylococcus aureus* ATCC 25923	—
Candida guilliermondii	+	*Enterobacter cloacae* ATCC 13047	—	*Staphylococcus epidermidis* ATCC 14990	—
Candida albicans	+	*Escherichia coli* ATCC 25922	—	*Streptococcus pyogenes* ATCC 10389	—
Candida crusei	+				

+ = inhibition zone >9 mm, — = no inhibition

References

1) WINKELMANN, G.; CH. GLÜCK, R. LUPP & G. JUNG: Herbicolins—New peptide antibiotics from enterobacteria. *in*: Structure and Activity of Natural Peptides, Selected topics. Proceedings of the Fall Meeting of Gesellschaft für Biologische Chemie held at Tübingen, Germany, September 1979 (W. VOELTER and G. WEITZEL, *eds*.), Walter De Gruyter, Berlin-New York, in press
2) VON ARX, E.; M. FAUPEL & M. BRUGGER: Das 4,4′-Tetramethyldiamino-diphenylmethan Reagens (TDM). Eine Modifikation der Chlor-*o*-Tolidin Farbreaktion für die Dünnschichtchromatographie. J. Chromatogr. 120: 224~228, 1976
3) TJOENG, F.-S.; E. KRAAS, E. STARK, E. BREITMAIER & G. JUNG: Vier Synthesewege zu (2-Pyrimidinylamino)-*n*-alkansäuren. Chem. Ber. 108: 862~874, 1975
4) FRANK, H.; G. J. NICHOLSON & E. BAYER: Rapid gas chromatographic separation of amino acid enantiomeres with a novel chiral stationary phase. J. Chromatogr. Sci. 15: 174~176, 1977